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IAPs limit activation of RIP kinases by TNF Receptor 1 during development

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 November 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see while referee 1 is more positive and all three referees consider the study as interesting, referees 2 and 3 are concerned that the rescue effect in the triple KO mice with the RIPKs is only partial and that thus the contribution of RIPKs to the death of cIAP1/2 DKO mice is relatively minor. Another, related concern raised by both referees is that there is too little insight into why IAP-deficient mice die and whether this is indeed due to cardiovascular failure. Clearly, these connected aspects will need to be strengthened along the lines suggested by the referees and to their full satisfaction. We should thus be able to consider a revised manuscript in which these issues and the other points raised by the referees are addressed in an adequate manner.

I should add that it is EMBO Journal policy to allow only a single round of revision, and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as

soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This is an outstanding piece of work. It carefully addresses the perplexing interplay between mouse IAPs at the genetic level, and therefore adds substance to the previously determined biochemical and cell biology studies. Given the recent papers on the rescue of the caspase-8 defect in mice by ablation of RIPKs, the results presented in this paper are both timely and satisfying. There are no additional experiments required in my opinion. But there are some clarifications that need to be made before publication.

Main Points.

1. One has the impression from reading this manuscript that cIAPs and XIAP have similar mechanisms. This is incorrect. A plethora of biochemical and cell biological evidence clearly demonstrates that cIAPs regulate the TNFR1 complex signals, whereas XIAP regulates downstream caspase inhibition. The mouse genetics described in the manuscript is just too coarse to allow distinction of these issues, and so the manuscript must be altered to ensure that readers do not take home the wrong message. Indeed, so far as I read this manuscript, all of the phenotypes are explained by cIAPs regulating TNF/RIPK signals, and XIAP by regulating caspase signals.

2. Fig S4 is too informative for the supplemental material, and should be included in the main figures. To save space I recommend demoting Figs 1B and Fig 7 to the supplemental section.

3. I just finished supervising a graduate level scientific paper critique class, and there was an almost unanimous agreement that a (cartoon) model is very useful in papers such as this that seek to demonstrate interactions in a protein-interaction pathway. Please consider adding a model, and again demoting some figures to the supplemental section.

4. Page 12. The authors appear to have mis-interpreted a main message of the Oberst paper on caspase-8/RIPK3 double knockouts. In this paper it was suggested that FLIP is not anti-apoptotic, but pro-survival, a very different mechanism. Please correct the "general" thinking here.

Other points.

Fig 4. The variable use of color and grayscale in the panels is distracting.

Abbreviations - please define QVD (correct chemical name).

Fig 3 legend has a strange rectangular symbol in it.

Page 13 - the article "it" in the sentence "when normal levels of cIAP1 are present, it causes a high level of RIPK1 ubiquitylation, so that it activates p65/RelA NF-κB, but is then degraded and no cell death occurs;" is confusing. "It" may apply to TNF or cIAP1. Please re-phrase.

Figures - if the authors use a serif font for "cIap" it will look more like a gene name and less like a

disease.

Referee #2

Manuscript by Moulin et al reports double knockout (DKO) of cIap1 and cIap2 genes and examines the role of cIap genes in the context of TNF signaling pathways. The authors claim that IAPs must limit activation of RIPK proteins by TNFR1. This is based on crosses between of cIap1/cIap2 and cIap1/Xiap knockout mice with Ripk1, Ripk3, or Tnfr1 knockout animals.

However, the use of the term "rescue" is an overstatement because essentially all the animals die prior to weaning, and most prior to birth. This is in stark contrast to caspase-8/Ripk3 double knockout mice where deletion of Ripk3 gene indeed rescued lethal phenotype of caspase-8 knockout (Nature, 2011).

Tnfr1 deficiency delays death of the cIap1/2 DKO until just after birth. The authors mention in the discussion that this reflects either that TNFR1 signaling normally occurs as early as E10.5 or that cIap loss causes aberrant TNF production during development. The latter scenario predicts that elevated TNF might be detected in the DKOs so I wonder if they tried to measure TNF in cIap1/2-/- TNFR1-/- embryos vs. WT embryos, say at E18.5? This would be very informative.

Their main catch phrase, however, is that TNFR1 activates the RIPKs. Here their data is difficult to interpret:

1. Ripk1 heterozygosity is shown in Fig. 6C to allow Xiap/cIap1 DKOs to make it to weaning (note, however, that this panel is not referenced in the main text). What doesn't make sense is that complete loss of RIP1 doesn't appear to delay death until birth, which is when RIP1 deficiency alone is reported to be lethal. Specifically, resorption and defects are noted in 3 TKO embryos between E14.5 and E16.5. The authors should comment on why they think complete loss of RIP1 doesn't delay death until after birth.

2. Ripk1 heterozygozity and Ripk1 deficiency also are claimed to delay death of cIap1/cIap2 DKO embryos to varying degrees (reflected in Fig. S4). Perhaps this variability, and even the claimed "rescue" by a few days, just stems from analyzing small numbers of embryos and is not statistically significant?

3. The authors have not presented convincing evidence that cIap1/2 DKO embryos are dying of cardiovascular failure. Caspase-8 KO animals reportedly had similar phenotype but they were efficiently rescued by crossing to Ripk3 KO animals. Ripk3 deficiency offers minimal benefit to cIap1/2 DKO embryos, which is quite different to the studies involving FADD or Casp8 KO mice. What is the explanation and how does the absence of Ripk1 or Ripk3 affect cardiovascular defects?

4. I was under impression that the authors already reported the importance of cIap1 for TNF-induced apoptosis in MEFs (Cell, 2007). Thus, it is not clear why they re-examine TNF-induced signaling in MEFs. It would be much more informative if they examined wider variety of cell lines, not just MEFs, and focus more on the cause of death of cIap1/2 DKO embryos. Related to this point, RIPK1 is still ubiquitylated in figure 6B in Xiap/cIap1 DKO MEFs. Is this abnormal ubiquitylation since the authors state on page 10 that "normal ubiquitylation of RIPK1 was observed only when cIAP1 was present"?

Specific points:

- The manuscript contains some strange math; e.g. Fig 5A, top row of table: since when does 42 + 78 = 114?
- Also, where are the single KO controls for antibody specificity in Fig. 1D?

Referee #3

Knock out mice lacking cIAP1, cIAP2 or xIAP have no immediately obvious phenotype. There is,

however, evidence from the combined use of knock out cells, RNA interference and cIAP-degrading reagents that these molecules act at least partly redundantly in the inhibition of the alternative NFkappaB pathway and TNF-induced cell death and also functions in TNFR1-mediated activation of the classical NFkappaB pathway.

Moulin et al present here a systematic genetic analysis of mice (and MEFs) lacking any two-part combination of these three IAPs. The authors found i) that xIAP/cIAP1 and cIAP1/cIAP2 double deficient mice are embryonic lethal with a phenotype resembling those of FADD, Caspase-8 and FLIP deficient mice while xIAP/cIAP2 double deficient mice are viable ii) that cIAP1 is necessary and sufficient for TNFR1-induced activation of the classical NFkappaB pathway iii) that cIAP1 is sufficient to inhibit p100 processing and iv) that TNFR1, RIPK1 or RIPK3 deficiency or RIPK1 haplodeficiency delay the deadly phenotype of xIAP/cIAP1 and cIAP1/cIAP2 double deficient mice.

The experiments presented are comprehensive, straight forward, technical sound and of broad interest. Particularly, the study by Moulin et al complements very well with some recent reports demonstrating that RIPK1 or RIPK3 deficiency partly rescues/delay the embryonic lethality of FADD, Caspase-8 and FLIP deficient mice.

I have only some minor comments:

Figure 3 A: A band migrating between the p52 and p100 bands of NFkappaB2/p100 is labelled as non-specific. This is not really convincing because the intensity of this band is not the same in all lanes and instead correlates with the increase in p52. Thus, is this band also detectable with other independent p100-specific antibodies?

Figure 5C: TNFR2 is mainly expressed in immune cells. It is therefore not trivial that there is TNFR2 expression in MEFs. To make clear that the MEFs used indeed express this receptor type corresponding FACS data should be performed/included.

RIPK1 or RIPK3 deficiency delay but do not prevent lethality associated with xIAP/cIAP1 and cIAP2/cIAP1 double deficiency. Display all mice a cardiac-related haemorrhagic phenotype at the time of death? Is there evidence for an increase in apoptotic cells in the heart of in the triple deficient mice?

The authors should discuss the unexpected observation that RIPK1 haplodeficiency prolongs the survival of xIAP/cIAP1 double deficient embryos better than complete RIPK1 deficiency.

1st Revision - authors' response 20 December 2011

Referee #1

This is an outstanding piece of work. It carefully addresses the perplexing interplay between mouse IAPs at the genetic level, and therefore adds substance to the previously determined biochemical and cell biology studies. Given the recent papers on the rescue of the caspase-8 defect in mice by ablation of RIPKs, the results presented in this paper are both timely and satisfying. There are no additional experiments required in my opinion. But there are some clarifications that need to be made before publication.

Main Points.

1. One has the impression from reading this manuscript that cIAPs and XIAP have similar mechanisms. This is incorrect. A plethora of biochemical and cell biological evidence clearly demonstrates that cIAPs regulate the TNFR1 complex signals, whereas XIAP regulates downstream caspase inhibition.

We agree; we did not intend to give the impression that cIAPs and XIAP have the same functions, and believe some of the functions are unique, and some are shared.

The mouse genetics described in the manuscript is just too coarse to allow distinction of these issues, and so the manuscript must be altered to ensure that readers do not take home the wrong message. Indeed, so far as I read this manuscript, all of the phenotypes are explained by cIAPs regulating TNF/RIPK signals, and XIAP by regulating caspase signals.

This is one possible explanation for the phenotypes, but the model we favor is that while cIAP1 can fulfil all the functions on its own (XIAP,cIAP2 DKOs, which only have cIAP1, are ~normal), cIAP2 is not present at high enough levels to do the job, unless XIAP is also present to 'help' it. This 'help' might be by inhibiting caspases, but it also might be by binding to TAB1 or by sequestering BIR-binding proteins (such as smac/diablo) away from cIAP2.

We have expanded the discussion to mention these possibilities. (Discussion, paragraph 7).

2. Fig S4 is too informative for the supplemental material, and should be included in the main figures. To save space I recommend demoting Figs 1B and Fig 7 to the supplemental section.

We have made Fig S4 into Figure 8A.

3. I just finished supervising a graduate level scientific paper critique class, and there was an almost unanimous agreement that a (cartoon) model is very useful in papers such as this that seek to demonstrate interactions in a protein-interaction pathway. Please consider adding a model, and again demoting some figures to the supplemental section.

We have made Fig S4 into Figure 8A, and also added a cartoon model Fig. 8B.

4. Page 12. The authors appear to have mis-interpreted a main message of the Oberst paper on caspase-8/RIPK3 double knockouts. In this paper it was suggested that FLIP is not anti-apoptotic, but pro-survival, a very different mechanism. Please correct the "general" thinking here.

Yes, good point; we have changed the wording accordingly.

Other points.

Fig 4. The variable use of color and grayscale in the panels is distracting.
We have made all the figures grayscale.

Abbreviations - please define QVD (correct chemical name).
Done.

Fig 3 legend has a strange rectangular symbol in it.
Fixed.

Page 13 - the article "it" in the sentence "when normal levels of cIAP1 are present, it causes a high level of RIPK1 ubiquitylation, so that it activates p65/RelA NF-κB, but is then degraded and no cell death occurs;" is confusing. "It" may apply to TNF or cIAP1. Please re-phrase.
We agree – we have rephrased it.

Figures - if the authors use a serif font for "cIap" it will look more like a gene name and less like a disease.

Referee #2

Manuscript by Moulin et al reports double knockout (DKO) of cIap1 and cIap2 genes and examines the role of cIap genes in the context of TNF signaling pathways. The authors claim that IAPs must limit activation of RIPK proteins by TNFR1. This is based on crosses between of cIap1/cIap2 and cIap1/Xiap knockout mice with Ripk1, Ripk3, or Tnfr1 knockout animals.

This conclusion is also based on results from the TNFR2 KO crosses.

However, the use of the term "rescue" is an overstatement because essentially all the animals die prior to weaning, and most prior to birth. This is in stark contrast to caspase-8/Ripk3 double knockout mice where deletion of Ripk3 gene indeed rescued lethal phenotype of caspase-8 knockout (Nature, 2011).

Point taken (although the XIAP-/- cIAP2-/- mice make it to adulthood, as have some XIAP-/- cIAP1-/- RIPK1+/- mice). We have changed the text so that whenever we use the word "rescue" or the term "prolong survival" we include a qualifier, such as "to birth" or "until E14" to indicate precisely when death occurs.

Tnfr1 deficiency delays death of the cIap1/2 DKO until just after birth. The authors mention in the discussion that this reflects either that TNFR1 signaling normally occurs as early as E10.5 or that cIap loss causes aberrant TNF production during development. The latter scenario predicts that elevated TNF might be detected in the DKOs so I wonder if they tried to measure TNF in cIap1/2-/- TNFR1-/- embryos vs. WT embryos, say at E18.5? This would be very informative.

TNF has already been shown to be expressed at E14.5 in WT embryos (PNAS 96 2994–2999 (1999)), so not much would be gained by showing that it is present at E18.5. It would be interesting to do the experiment at E10, but this is technically much more challenging, as the embryos are much smaller, and this experiment could not be completed within 3 months.

Their main catch phrase, however, is that TNFR1 activates the RIPKs. Here their data is difficult to interpret:
1. Ripk1 heterozygosity is shown in Fig. 6C to allow Xiap/cIap1 DKOs to make it to weaning (note, however, that this panel is not referenced in the main text).

We have corrected this typo where we incorrectly referred to Fig. 7 instead of Fig. 6C.

What doesn't make sense is that complete loss of RIP1 doesn't appear to delay death until birth, which is when RIP1 deficiency alone is reported to be lethal. Specifically, resorption and defects are noted in 3 TKO embryos between E14.5 and E16.5. The authors should comment on why they think complete loss of RIP1 doesn't delay death until after birth.

We have now added a figure with a model (Fig. 8), and speculated on why heterozygosity for RIPK1 allows longer survival than RIPK1 +/+ or -/- on a cIAP1/cIAP2 DKO background.

Briefly, we believe it is because cIAPs not only inhibit the killing function of the RIP kinases, but also act together with RIPK1 to fully activate canonical NF-kB. If both copies of RIPK1 are present, the cIAP1/cIAP2 and cIAP1/XIAP DKO mice die at E10 from uncontrolled RIPK1-dependent death. If no RIPK1 is present, the cIAP1/cIAP2/TNFR1 TKO mice die at E12.5 and the XIAP/cIAP1/RIPK1 TKO mice might die at E15-16 due to insufficient NF-kB signalling (as do p65/RelA KOs).

2. Ripk1 heterozygozity and Ripk1 deficiency also are claimed to delay death of cIap1/cIap2 DKO embryos to varying degrees (reflected in Fig. S4). Perhaps this variability, and even the claimed "rescue" by a few days, just stems from analyzing small numbers of embryos and is not statistically significant?

Yes, our numbers are too small to make definitive conclusions about the differences between the IAP DKO RIPK1 heterozygous vs homozygous mice, but, because we have had no cIAP1/cIAP2 DKOs or cIAP1/XIAP DKOs surviving past E10, but many do if we also knock out TNFR1, RIPK1, or RIPK3, these results are statistically significant.

We have now added a figure with a model (Fig. 8), and speculated on why heterozygosity for RIPK1 allows longer survival than RIPK1 +/+ or -/- on a cIAP1/cIAP2 DKO background.

3. The authors have not presented convincing evidence that cIap1/2 DKO embryos are dying of cardiovascular failure. Caspase-8 KO animals reportedly had similar phenotype but they were efficiently rescued by crossing to Ripk3 KO animals. Ripk3 deficiency offers minimal benefit to

cIap1/2 DKO embryos, which is quite different to the studies involving FADD or Casp8 KO mice. What is the explanation and how does the absence of Ripk1 or Ripk3 affect cardiovascular defects?

On the contrary, deletion of RIPK3 prevented the death at E10 with the *tomato* phenotype, and allowed development to proceed until day E14. One explanation might be that during development FADD, FLIP, caspase 8 and IAPs all suppress (directly or indirectly) activity RIP kinases, but unlike FADD, FLIP and caspase 8, IAPs also have functions later in development, such as regulating transcription factors. We have tried to explain this in the model in the new Fig. 8.

4. I was under impression that the authors already reported the importance of cIap1 for TNF-induced apoptosis in MEFs (Cell, 2007). Thus, it is not clear why they re-examine TNF-induced signaling in MEFs. It would be much more informative if they examined wider variety of cell lines, not just MEFs, and focus more on the cause of death of cIap1/2 DKO embryos. Related to this point, RIPK1 is still ubiquitylated in figure 6B in Xiap/cIap1 DKO MEFs. Is this abnormal ubiquitylation since the authors state on page 10 that "normal ubiquitylation of RIPK1 was observed only when cIAP1 was present"?

The reviewer is correct that we previously reported the importance of cIAP1 for preventing TNF inducing apoptosis of MEFs, but this earlier paper described the effects of cIAP1 *single* KOs and smac mimetic, whereas the current manuscript describes the effects of *double* IAP KOs. Examining a variety of further types of cells will be interesting, but is beyond the scope of this paper. (Although those interested in the effects of deletion of both cIAP1 and cIAP2 in B cells can read the paper in Blood 117: 4041-4051.) When we referred to “normal ubiquitylation” we mean normal *levels* of ubiquitylation. We have modified the text to make this clearer.

Specific points:

- The manuscript contains some strange math; e.g. Fig 5A, top row of table: since when does 42 + 78 = 114?
Thank you for picking up this error. We have corrected the maths.

- Also, where are the single KO controls for antibody specificity in Fig. 1D?
We used the same antibodies on extracts from KO cells for Fig. 3A, which shows that they are specific.

Referee #3

Knock out mice lacking cIAP1, cIAP2 or xIAP have no immediately obvious phenotype. There is, however, evidence from the combined use of knock out cells, RNA interference and cIAP-degrading reagents that these molecules act at least partly redundantly in the inhibition of the alternative NFkappaB pathway and TNF-induced cell death and also functions in TNFR1-mediated activation of the classical NFkappaB pathway.

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I have only some minor comments:

Figure 3 A: A band migrating between the p52 and p100 bands of NFkappaB2/p100 is labelled as non-specific. This is not really convincing because the intensity of this band is not the same in all lanes and instead correlates with the increase in p52. Thus, is this band also detectable with other independent p100-specific antibodies?

This band is carry over signal from the previous probing for NIK. The antibody to NIK detects a number of proteins non-specifically in addition to NIK. We have therefore changed the legend to indicate that the band is a non-specific band from previous probing with the NIK antibody, so that readers will know it is not related to NFkappaB2/p100.

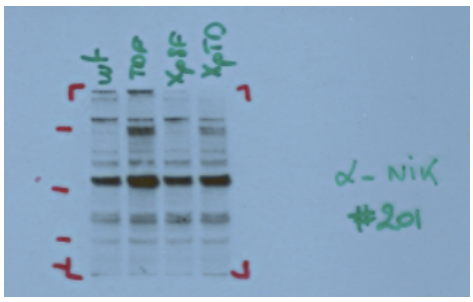

Figure 5C: TNFR2 is mainly expressed in immune cells. It is therefore not trivial that there is TNFR2 expression in MEFs. To make clear that the MEFs used indeed express this receptor type corresponding FACS data should be performed/included.

We agree with the reviewer. TNFR2 is expressed on immune cells and not on MEFs, and we were *not* able to detect it on MEFs by FACS (Wong et al CDD (2010) 17, 482–487). The fact that the TNFR2 KO MEFs behave the same way as WT MEFs is consistent with this. We have changed the labelling on Fig. 5C as we think this might have confused the reviewer.

RIPK1 or RIPK3 deficiency delay but do not prevent lethality associated with xIAP/cIAP1 and cIAP2/cIAP1 double deficiency. Display all mice a cardiac-related haemorrhagic phenotype at the time of death? Is there evidence for an increase in apoptotic cells in the heart of in the triple deficient mice?

Only the mice that die at E10 show the *tomato* phenotype. In the triple deficient mice (which don't die at E10, and don't show the *tomato* phenotype) we have not seen any abnormalities in the hearts. Our current hypothesis is that those mice that die later in development or perinatally might succumb from liver disease rather than anatomical heart defects, and our preliminary analysis has shown a small liver in the cIAP1/CIAP2/RIPK3 TKO embryo. We hope to include a more thorough analysis including results of more crosses (such as TNFR1/casp8 DKOs) in a subsequent manuscript.

The authors should discuss the unexpected observation that RIPK1 haplodeficiency prolongs the survival of xIAP/cIAP1 double deficient embryos better than complete RIPK1 deficiency.

We have included a model (new Fig. 8) and discussed this in the Discussion.

2nd Editorial Decision 04 January 2012

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, you may wish to consider addressing the minor points suggested by referee 2 (see below) by additional text changes. Furthermore, I would like to urge you to work a bit more on the title. One possibility could be to simply invert the sentence to 'IAPs must limit activation of RIP kinases by TNF Receptor 1 to prevent embryonic lethality'.

As a new policy, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number, and should have molecular weight markers;

further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

The authors have largely addressed the criticism from three reviewers and, importantly, they now define the outcome of triple knockouts in a more precise fashion that clearly adds to the accuracy of their study.

I have only few minor points that still have to be addressed, mainly by rewording.

- The authors have clarified the role of XIAP much better but I do not think they need to mention binding of XIAP to TAB1 in their Discussion. This current study is a nice example of how physiological data can support the relevance of the presumed role of a gene/protein. It is clear from this and many other studies that there is no physiological significance for endogenous XIAP-TAB1 interaction, especially for NF-kB or MAPK activation. Thus, this statement does not add any value and should be omitted.

- Similarly, added comment about ripoptosome on page 15 is also not needed as this proposed ripoptosome complex was only shown in few cancer cell lines but not in murine tissues or normal fibroblasts.

- Also on page 15, it is not clear how do "cIAPs inhibit cell death by ... and by reducing activation of non-canonical (NIK-dependent) NF-kB." Why would activation of non-canonical NF-kB activate cell death but activation of canonical NF-kB inhibit cell death? Maybe it would be better to state: "cIAPs regulate cell death by...".

- Finally, on page 3 in the Introduction the authors should add appropriate references where needed, which is not case at the moment. For example, in the first paragraph the authors list different protein domains of IAP proteins but only provide a reference for the UBA domain. What about other domains (BIRs, RING)?

Referee #3

I considered already the first version of the manuscript as a quite good piece of work, well suited for the EMBO Journal. I had only a few minor comments/concerns that were all satisfactorily addressed by small modifications in the revised version or by clarification in the response letter. I think the authors have also responded well to the issues raised by the other reviewers, particular those of the a bit more critical reviewer 2.

2nd Revision - authors' response 08 January 2012

Reviewer #2 has made some further suggestions just regarding the text:

The authors have largely addressed the criticism from three reviewers and, importantly, they now define the outcome of triple knockouts in a more precise fashion that clearly adds to the accuracy of their study.

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We added these comments for reviewer #1, who asked us to state how XIAP might be acting other than just by inhibiting caspase activity. The XIAP-TAB1 interaction was the basis of a paper in Molecular Cell, and we have no reason to doubt its claims.

- Similarly, added comment about ripoptosome on page 15 is also not needed as this proposed ripoptosome complex was only shown in few cancer cell lines but not in murine tissues or normal fibroblasts.

It does not strike us as being unreasonable to mention results involving the same components that have been described in multiple cell types. I think it is only a matter of time before the ripoptosome is shown to exist in mouse cells.

- Also on page 15, it is not clear how do "cIAPs inhibit cell death by ... and by reducing activation of non-canonical (NIK-dependent) NF-kB." Why would activation of non-canonical NF-kB activate cell death but activation of canonical NF-kB inhibit cell death? Maybe it would be better to state: "cIAPs regulate cell death by...".

To state “cIAPs regulate cell death…” rather than “cIAPs inhibit cell death…” introduces an unnecessary vagueness, because cIAPs have never been shown to promote cell death. Maybe non-canonical NF-kB stimulates autocrine production of TNF more efficiently than canonical NF-kB.

- Finally, on page 3 in the Introduction the authors should add appropriate references where needed, which is not case at the moment. For example, in the first paragraph the authors list different protein domains of IAP proteins but only provide a reference for the UBA domain. What about other domains (BIRs, RING)?

We have added a reference to these domains.

